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# Determination of Ractopamine in Cattle and Sheep Urine Samples Using an Optical Biosensor Analysis: Comparative Study with HPLC and ELISA

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A biosensor method, using the surface plasmon resonance (SPR) principle, was developed for the determination of ractopamine in cattle and sheep urine. A monoclonal antibody was used to compete with ractopamine in the sample and ractopamine immobilized on the sensor chip. Addition of bovine serum albumin (BSA, 1 mg/mL) as an antibody stabilizer to the incubation buffer was required to achieve a stable biosensor response throughout each sample set. The calibration curve gave a mean $IC_{50}$ of 4.7 ± 0.21 ng/mL ($n = 7$). Over sample concentrations from 2.5 to 10 ng/mL recoveries were typically ~100−110%, whereas inter- and intra-assay reproducibilities (% CV) were usually less than 10 and 6%, respectively. Comparison of biosensor results with results obtained from high-performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assays (ELISA) using enzyme-hydrolyzed urine (to convert ractopamine conjugates to free ractopamine) gave correlation coefficients of 0.94 for sheep and 0.86 for cattle. Slopes of the lines, with zero intercepts, equaled 0.80 for sheep and 0.74 for cattle. For untreated (nonhydrolyzed) urine samples, the correlations between biosensor and HPLC results were 0.95 for sheep and 0.72 for cattle with slopes of 1.18 (sheep) and 1.69 (cattle). The slopes greater than unity indicate that the biosensor responded to ractopamine metabolites in addition to free ractopamine. The biosensor assay is an excellent analytical tool to screen ractopamine residues in sheep or cattle urine, and the results should be extendible to other species with suitable validation.

**KEYWORDS: Biosensor; immunosensor; ractopamine; ELISA; HPLC; analysis; residue; measurement; SPR**

## INTRODUCTION

Ractopamine is a β-adrenergic agonist leanness-enhancing agent that was recently approved by the U.S. Food and Drug Administration for use as a swine feed additive (*1*, *2*). Hogs fed ractopamine at a dietary level of 20 ppm reached target market weights an average of 4 days earlier, have leaner carcasses, and consume less feed than similarly managed control animals (*3*). The economic advantage of ractopamine use in swine could lead animal producers to use ractopamine in species for which no approval exists. Mitchell and Dunnavan have reported that β-adrenergic agonists have been used illicitly in show animals within the United States (*4*). Because of a potential for illegal ractopamine use, and because the European Union has banned the use of growth promoters (including all β-agonists) in farm animals, there is a need for rapid detection methods of animal exposure to ractopamine.

The official regulatory determinative (tissue) method of ractopamine analysis requires multiple extractions (liquid−liquid partition and solid phase) followed by high-performance liquid chromatography (HPLC) analysis with fluorescence detection (method B03766; available from the U.S. FDA upon request) (*5*). To the authors' knowledge a rapid screening method approved by regulatory officials does not exist. Immunoassays have the advantages of high throughput, rapid turnaround time, user friendliness, and field portability. Previously, our laboratory has developed polyclonal and monoclonal immunoassays for ractopamine having sensitivities in the low parts per billion range (*6*, *7*). The monoclonal antibody based immunoassay has been validated with regard to sensitivities and cross-reactivities and has been tested for its utility in samples from cattle and sheep. We found that when measurements of ractopamine were made on enzyme-hydrolyzed urine samples, the enzyme-linked immunosorbent analysis (ELISA) method yielded results that were highly correlated with results obtained by an HPLC method (*8*).

Optical biosensor analysis using surface plasmon resonance (SPR) has been used by a number of investigators for residue analyses (*9*). Briefly, SPR causes a reduction in the amount of light reflected at a given angle from a conducting interface of media of a different refractive index. The binding of a molecule

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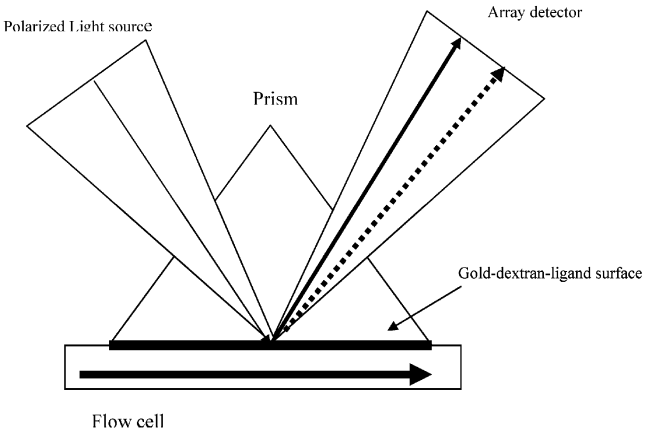


**Figure 1.** Basic surface plasmon resonance instrumentation. Polarized light impinges on a prism in which one side (the flow cell side) is coated with gold and a dextran surface to which is attached a receptor. When the analyte passes by the antibody, it binds, changing the refractive index that changes the angle at which the resonance occurs, shifting the angle of maximum absorbance of energy detected by the array detector. The result is displayed as a sensorgram.

to the chip surface changes the angle of light reflected from the chip, and this change in reflectance is detected and measured (**Figure 1**). For optical biosensor applications, an analyte is covalently attached to a sensor chip and an antibody in the flow system binds to the analyte on the chip. When a sample is introduced containing the free analyte, it competes for the antibody with the analyte covalently bound to the sensor chip and the light angle detected by the sensor will change. The response change is inversely proportional to the analyte concentration in the sample. Thus, with the use of analytical standards a quantitative relationship between reflectance angle and analyte concentration may be established. Fully automated optical biosensors have been developed, allowing for the unattended analysis of sample sets; the technique has been applied to residues analysis in a variety of matrices (*10*−*15*). This recent analytical technology offers some advantages over conventional analysis. A major advantage of SPR optical biosensors over conventional analytical techniques is the minimal matrix effect on the detector response, a factor of considerable importance in the measurement of residues in foods and other complex biological matrices. The objective of this study was to assess the utility of an optical biosensor analysis of ractopamine by comparison with results obtained from HPLC and ELISA analyses of the same samples.

## MATERIALS AND METHODS

**Materials and Instrumentation.** Ractopamine (Paylean) was a gift from Lilly Research Laboratories Elanco Animal Health, Greenfield, IN. Sensor chips (CM5 research grade) were purchased from Biacore Inc. (Uppsala, Sweden). Buffer (HBS-EP) composed of 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.05% of Tween 20 was passed through a 0.22 $\mu$m filter and stored at 4 °C until used. *N*-Hydroxysuccinimide (NHS), *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide (EDC), and ethanolamine were purchased from Sigma-Aldrich (St. Louis, MO). The ractopamine derivative, immobilized onto the CM-5 sensor chip, was kindly provided by Dr. Simon Haughey, Xenosense Inc. (Belfast, Northern Ireland). The ractopamine monoclonal antibody (5G10) was generated previously in our laboratory (*8*).

A Biacore Q (Biacore AB) SPR instrument incorporated with control software (version 2.1) was utilized to determine the ractopamine concentration via SPR.

**Ractopamine Sensor Chip Preparation.** *Surface Preparation Unit Method.* Sensor chips (CM-5, which contains a carboxymethylated dextran matrix on a thin gold surface) were activated by injecting a solution containing 200 mM EDC/50 mM NHS into the flow cell. The solution was allowed to flow at 10 $\mu$L/min for a total of 7 min. After activation of the chip, the ractopamine derivative was injected at a flow rate of 5 $\mu$L/min for a 15-min period. Unreacted sites were then blocked using 1 M ethanolamine at a flow rate of 10 $\mu$L/min for 7 min.

*Manual Method.* Sensor chips CM-5 were placed individually in a Petri dish on a 3-D rotator (Lab-line Instruments, Inc., Melrose Park, IL), and reactions were performed at room temperature. The chip surface was wetted with 70 $\mu$L of water and incubated for 30 min, followed by two 15-min incubations with 70 $\mu$L of fresh 200 mM EDC/50 mM NHS. This was followed by two incubations with ractopamine conjugate (70 $\mu$L and 2 h each). Unbound sites on sensor chips were blocked with 70 $\mu$L of 1 M ethanolamine (30 min).

**Checkerboard Experiment.** Ractopamine monoclonal antibody was diluted in HBS-EP buffer (1:200, 1:500, 1:1000, 1:2000, and 1:5000). Various ractopamine concentrations (0, 1, 5, and 10 ng/mL in HBS-EP buffer) were tested in combination with the monoclonal antibody dilutions to determine the best concentration of antibody for the competition binding assays in the biosensor.

**Determination of Antibody Stability.** Ractopamine monoclonal antibody 5G10 (*7*) was diluted 1:2000 with HBS-EP buffer or with HBS-EP containing 1 mg/mL BSA. To determine whether baseline shifts or changes in response to standards occurred over the duration of the run, the immunosensor response was monitored continuously, over the 16-h run (room temperature).

**Determination of Maximum Binding Capacity.** Mouse ascites fluid containing ractopamine monoclonal antibody (1:2, 1:5, 1:10, 1:20, 1:50, and 1:100 dilutions in HBS-EP or HBS-EP + 1 mg/mL BSA) were injected onto the ractopamine sensor chip at 5 $\mu$L/min for 15 min, and the relative response was measured. This flow rate was selected to ensure maximum binding. Responses were based on the difference in signal 10 s before and 30 s after injection of diluted ascites fluid. Sensor chips were regenerated by injecting 0.1 N NaOH in 20% $CH_3CN$ at a flow rate of 20 $\mu$L/min for 1.5 min; three regeneration cycles were run between samples.

**Urine Samples.** Urine samples containing ractopamine residues were collected from cattle and sheep fed a diet containing 20 ppm of ractopamine for 7 or 8 consecutive days, respectively (*16*). Urine was collected during the feeding and withdrawal periods. Samples were stored at −20 °C until analysis.

Sample preparation techniques utilized prior to HPLC or ELISA analyses were reported previously (*8*, *16*). For this study, raw urine samples were diluted with HBS-EP for immunosensor analysis or with 100 mM phosphate buffer containing 0.05% Tween 20 and 10 ng/mL of BSA for ELISA measurements. Alternatively, urine samples were diluted 1:1 with ammonium acetate buffer (1 M, pH 5.0) and then enzymatically hydrolyzed overnight (37 °C) with 5000 Fishman units of glucuronidase/arylsulfatase (from *Patella vulgata*). Samples were then prepared for analysis as described by Smith and Shelver (*16*). Briefly, the ractopamine freebase was formed with addition of 2 M sodium carbonate buffer and extracted with ethyl acetate. The solvent was evaporated using a centrifugal evaporator (Savant, Holbrook, NY), and ractopamine was converted to the HCl salt by reconstitution in 50 mM HCl. Samples were further purified by passing the solution through a C-18 solid phase extraction (SPE) column washed with 50% MeOH/$H_2O$, and ractopamine was eluted with 50% MeOH/0.05 M ammonium acetate buffer (pH 4.5).

**Biosensor Analysis.** Ascites fluid generated from monoclonal antibody 5G10 (*7*) (1:2000 dilutions into HBS-EP buffer in the presence of 1 mg/mL BSA) was mixed with samples or ractopamine standards at a 40:60 ratio. The solution was passed through the ractopamine sensor chip at flow rate of 20 $\mu$L/min for 6 min, and then the reaction was stopped and the sensor surface regenerated by the addition of 0.1 N NaOH/20% acetonitrile at flow rate of 20 $\mu$L/min for 1.5 min. These conditions are in the normal range of those reported for SPR experiments and were not optimized further because minimal improvement would be expected. With increasing ractopamine concentrations fewer antibodies are bound to the sensor, causing the detector response to be inversely proportional to ractopamine concentration. The concentration of ractopamine in urine samples was determined from a

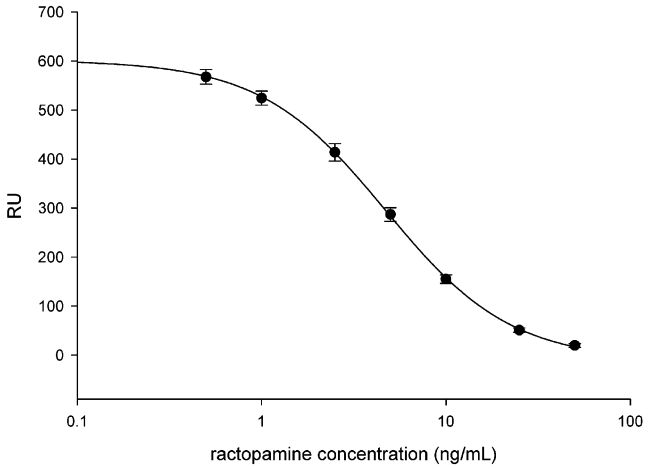


**Figure 2.** Ractopamine calibration curve obtained using the optical immunosensor and a monoclonal antibody against ractopamine (1:2000 in HBS-EP). An $IC_{50}$ of 4.7 (± 0.21) ng/mL was calculated from the curve ($n = 7$).

**Table 1.** Intra- and Inter-assay Variation of Sheep and Cattle Urine Spiked with Ractopamine

| | inter-assay[a] | | | | intra-assay[b] | | | |
|---|---|---|---|---|---|---|---|---|
| level, ng/mL | *n* | measured, ng/mL | recovery, % | CV, % | *n* | measured, ng/mL | recovery, % | CV, % |
| | | | | Sheep Urine | | | | |
| 1 | 5 | 1.31 ± 0.25 | 130.6 | 19.1 | 8 | 0.99 ± 0.03 | 98.7 | 3.0 |
| 2.5 | 5 | 2.73 ± 0.23 | 109.2 | 8.3 | 8 | 2.50 ± 0.05 | 100 | 2.0 |
| 5 | 5 | 5.08 ± 0.24 | 101.6 | 4.7 | 8 | 4.96 ± 0.29 | 99.2 | 5.8 |
| 10 | 5 | 10.67 ± 0.28 | 106.7 | 2.6 | 8 | 10.5 ± 0.14 | 105 | 1.3 |
| | | | | Cattle Urine | | | | |
| 1 | 5 | 1.02 ± 0.07 | 102 | 6.9 | 8 | 0.92 ± 0.03 | 91.6 | 2.7 |
| 2.5 | 5 | 2.45 ± 0.23 | 98 | 9.4 | 8 | 2.48 ± 0.08 | 99.2 | 3.2 |
| 5 | 5 | 5.04 ± 0.36 | 100.8 | 7.1 | 8 | 4.80 ± 0.11 | 96 | 2.3 |
| 10 | 5 | 10.88 ± 1.78 | 108.8 | 16.4 | 8 | 9.88 ± 0.92 | 98.8 | 9.3 |

[a] Inter-assay variation was determined by four replicates on five different days. [b] Intra-assay variation was determined by eight replicates on a single day.

calibration curve generated from ractopamine in HBS-EP at 0, 0.5, 1, 2.5, 5, 10, 25, and 50 ng/mL. The curve was fitted with a four-parameter logistic equation using Biacore control software. A calibration curve fitted to data obtained over seven separate runs is shown in **Figure 2**.

**Matrix Effects Determination.** Standard curves with final ractopamine concentrations of 0, 0.5, 1, 2.5, 5, 10, 25, and 50 ng/mL were run in the HBS-EP buffer and in various dilutions (1:2, 1:5, 1:10, and 1:20) of sheep or cow urine. The dilution at which the urine produced a standard curve with no significant difference from the standard curve carried out in the HBS-EP buffer was selected as the working dilution for the inter- and intra-assay determinations.

**Inter- and Intra-assay Variation Determinations.** Ractopamine was fortified to final concentrations of 1, 2.5, 5, and 10 ng/mL in sheep or cow urine diluted 1:5 or 1:10, respectively, with HBS-EP. Inter-assay variation was computed from the analysis of four replicates of each dilution carried out on five different days. Intra-assay variation was measured by analysis of eight replicates of each dilution on a single day. Sample recoveries were determined from a standard curve with ractopamine concentrations of 0, 0.5, 1, 2.5, 5, 10, 25, and 50 ng/mL in sheep urine diluted 1:5 with HBS-EP and in cow urine diluted 1:10 with HBS-EP. The results are shown in **Table 1**.

**ELISA and HPLC Measurements.** HPLC analysis of ractopamine in urine samples was described previously (*16*). Briefly, the HPLC system consisted of a Waters model 600E HPLC pump (Waters, Milford, MA) and a Jasco (Tokyo, Japan) model FP-920 fluorescence detector (excitation wavelength, 226 nm; emission wavelength, 305 nm). A Waters Symmetry column (4.6 × 250 nm, 5 um) was run with an isocratic mobile phase consisting of 5 mM sodium octanesulfonate in 2% acetic acid in water/acetonitrile (72:28) at a flow rate of 1 mL/min. Unknown concentrations of ractopamine were determined using ractopamine standard curves consisting of points at 50, 100, 200, 400, 800, 1600, and 3200 ng/mL (50-$\mu$L injections).

ELISA measurements of raw or enzyme-hydrolyzed cattle and sheep urine samples have been reported (*8*). In short, ELISA plates were coated with 500 ng/well of ractopamine−hemiglutarate−BSA for 2 h, and excess binding sites were blocked by 3% BSA for 1 h. Competitors were dissolved in 100 mM phosphate buffer containing 0.05% Tween 20 and 10 mg/mL BSA and co-incubated with primary antibody at 1:14000 dilution for 90 min. After incubation with hydrogen peroxidase labeled anti-mouse-IgG (1 h), the color was developed using 3,3′,5,5′-tetramethylbenzidine as substrate. A ractopamine calibration curve was used to determine the ractopamine level in the urine samples (0, 1, 2.5, 5, 7.5, 10, 25, 50, 75, 100, 250, and 500 ng/mL, added 100 $\mu$L/well).

## RESULTS AND DISCUSSION

The purpose of the present study was to develop a reproducible and stable assay for ractopamine using SPR optical biosensor technology and to compare optical biosensor results with results obtained from ELISA and HPLC analyses. Samples from a ractopamine feeding experiment (urine) were utilized in the study because they included a high concentration of metabolites (*8*, *16*) and other potentially interfering substances that would likely be encountered if the biosensor were used for ractopamine analysis. The samples were from two ruminant species for which ractopamine approvals have not currently been granted, namely, sheep and cattle.

When the ractopamine antibody used in the biosensor analysis was diluted with HBS-EP, the biosensor response was not stable at room temperature for 16 h. The addition of BSA (1 mg/mL) stabilized the antibody solution, enabling a stable detector response over a total of 70 analytical cycles. This observation is consistent with data generated by Gonzalez-Martinez et al. (*17*), who used BSA as a stabilizer for biosensor analysis of environmental contaminates.

Checkerboard dilution optimization of the five antibody dilutions tested indicated that 1:1000 and 1:2000 antibody dilutions were essentially identical in terms of the competition curves produced and the derived $IC_{50}$ values (data not shown). Therefore, 1:2000 antibody dilutions were used for subsequent studies.

Use of either the biosensor surface preparation unit or the manual method of binding analyte to the sensor surface produced similar detector responses. When the surface preparation unit of the biosensor is used, the reagent additions are automatic; however, use of the surface preparation unit consumes more reagent than did the manual method and could be used to prepare only one flow cell at a time. Because Baxter et al. (*10*) suggested that use of the surface preparation unit could cause the system to be contaminated with immobilizing agent, we utilized the manual method of chip preparation for most of our study.

Injection of excess antibody results in a maximum detector response ($B_{max}$) because the ractopamine binding sites on the chip are saturated with antibody. The $B_{max}$ for the ractopamine sensor chip, using a 1:5 dilution in HBS-EP, was 6109 ± 62 RU ($n = 3$); using HBS-EP + 1 mg/mL BSA, the $B_{max}$ was 6263 ± 131 RU ($n = 3$). These results demonstrate that BSA did not interfere with antibody binding. The immobilized sensor chip stability was demonstrated as more than 300 analysis cycles were run with no marked changes in the detector response.

The biosensor utilizes a competitive inhibition format and detects antibody bound to the immobilized ractopamine deriva-

**Table 2.** Comparisons of Ractopamine Quantitation by Immunosensor, ELISA, and HPLC (Nanograms per Milliliter) in Cow Urine Samples from a Ractopamine Feeding Study

| | cow 159 | | | | | average of all treated cows | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | raw diluted urine[a] | | hydrolyzed urine[b] | | | raw diluted urine[a] | | hydrolyzed urine[b] | | |
| day[c] | ELISA $\times 10^2$ | sensor $\times 10^2$ | ELISA $\times 10^2$ | sensor $\times 10^2$ | HPLC $\times 10^2$ | $n$ | ELISA[d] $\times 10^2$ | sensor $\times 10^2$ | ELISA[d] $\times 10^2$ | sensor $\times 10^2$ | HPLC[e] $\times 10^2$ |
| T1 | 121 | 49 | 27 | 29 | 29 | 6 | $73 \pm 50$ | $33 \pm 32$ | $19 \pm 13$ | $18 \pm 11$ | $18 \pm 12$ |
| T2 | 94 | 40 | 31 | 27 | 30 | 6 | $82 \pm 55$ | $35 \pm 28$ | $21 \pm 14$ | $18 \pm 13$ | $21 \pm 13$ |
| T3 | 107 | 63 | 32 | 29 | 32 | 6 | $138 \pm 69$ | $48 \pm 20$ | $36 \pm 18$ | $29 \pm 13$ | $38 \pm 18$ |
| T4 | 273 | 87 | 71 | 61 | 74 | 6 | $235 \pm 109$ | $83 \pm 52$ | $58 \pm 25$ | $42 \pm 20$ | $61 \pm 25$ |
| T5 | 198 | 109 | 55 | 48 | 59 | 6 | $225 \pm 62$ | $120 \pm 62$ | $60 \pm 16$ | $47 \pm 16$ | $63 \pm 17$ |
| T6 | 183 | 78 | 48 | 42 | 53 | 6 | $175 \pm 31$ | $67 \pm 33$ | $45 \pm 9.8$ | $33 \pm 6.8$ | $49 \pm 9.6$ |
| T7 | 77 | 60 | 43 | 37 | 42 | 6 | $191 \pm 87$ | $49 \pm 24$ | $52 \pm 13$ | $39 \pm 15$ | $50 \pm 16$ |
| W0 | 84 | 24 | 24 | 21 | 24 | 6 | $119 \pm 62$ | $60 \pm 30$ | $34 \pm 19$ | $30 \pm 17$ | $38 \pm 24$ |
| W1 | 27 | 21 | 8.4 | 7.7 | 10 | 4 | $74 \pm 41$ | $45 \pm 24$ | $21 \pm 11$ | $17 \pm 7.7$ | $25 \pm 14$ |
| W2 | 6.4 | 3.2 | 2.2 | 1.4 | 3.1 | 4 | $18 \pm 9.8$ | $7.9 \pm 3.7$ | $6.2 \pm 3.8$ | $5.3 \pm 3.2$ | $8.5 \pm 5.2$ |
| W3 | 1.3 | 0.47 | 0.27 | 0.28 | 0.53 | 4 | $8.8 \pm 5.7$ | $2.9 \pm 2.1$ | $2.5 \pm 1.9$ | $2.3 \pm 1.6$ | $4.3 \pm 3.3$ |
| W4 | 0.75 | 0.25 | 0.14 | 0.16 | 0.4 | 2 | 2.4 | 0.93 | 0.40 | 0.41 | 0.98 |
| W5 | 0.59 | 0.29 | 0.49 | 0.12 | <LOQ[f] | 2 | 1.2 | 0.48 | 0.43 | 0.23 | 0.71[g] |
| W6 | 0.39 | 0.22 | 0.04 | NA | <LOQ | 2 | 0.64 | 0.29 | 0.09 | <LOQ | <LOQ |
| W7 | 0.23 | 0.12 | 0.04 | NA | <LOQ | 2 | 0.31 | 0.13 | 0.06 | <LOQ | <LOQ |

[a] Raw urine samples were analyzed by cELISA and biosensor after dilution with variable amounts of 100 mM phosphate buffer containing 0.05% Tween 20 and 10 mg/mL BSA (ELISA) or HBS-EP (biosensor). [b] Urine samples were treated with enzyme to hydrolyze ractopamine conjugates, and samples were subsequently purified using liquid–liquid extraction and solid phase extraction techniques. [c] T represents day of treatment, and W represents day of withdrawal period. [d] ELISA data are from Shelver and Smith (*8*). [e] HPLC data are from Smith and Shelver (*16*). [f] LOQ for hydrolysis + SPE analyzed by HPLC was 50 ng/mL. [g] One sample was <LOQ.

tive on the sensor chip. The ractopamine calibration curve obtained from the biosensor analyses determined $IC_{50}$ values of $4.7 \pm 0.2$ ng/mL ($n = 7$) with a % CV of 4.4%, demonstrating the relative stability of the detector response to the standard curve.

Sheep urine, diluted 1:5, and cattle urine, diluted 1:10, in HBS-EP were chosen to perform the inter- and intra-assay variation tests (**Table 1**). These dilutions were selected because the resulting ractopamine standard curves had $B_0$ and $IC_{50}$ values similar to standard curves run in buffer. The similarity of the standard curves constructed in diluted urine versus buffer indicated that minimal matrix effects were present. The inter- and intra-assay variations for sheep and cattle urine are shown in **Table 1**. The recoveries and variation are quite acceptable with the potential exception of 1 ng/mL. The inter-assay variation for sheep urine was ~19%, and recovery was ~130% at 1 ng/mL concentration, which is at the lower end of the straight-line portion of the calibration curve and may explain the greater variance and high recoveries. The ractopamine biosensor assay was accurate and repeatable between 2.5 and 10 ng/mL.

The HPLC assay utilized in this study detected free ractopamine (parent ractopamine) with high sensitivity as a well-resolved peak using fluorescence detection. Use of the HPLC assay to quantify ractopamine in urine before and after hydrolysis of ractopamine conjugates with enzyme allowed us to validate the biosensor analysis with respect to free ractopamine in urine and with respect to the presence of ractopamine metabolites in urine samples. We also compared quantitative results obtained from the biosensor assay with quantitative results obtained by ELISA, an assay that we have previously reported to be sensitive to both ractopamine metabolites and individual ractopamine stereoisomers (*7*). Results of these comparisons are shown in **Tables 2** and **3** for a typical animal and for the average of six animals, respectively. Because both the biosensor and ELISA assays utilized the same antibody (5G10), comparison of the techniques allows an assessment of the assay format based on the quantitative results. Comparisons were made using linear regression analysis with the lines modeled having a zero intercept. The resulting correlation coefficients served as measures of assay variability, whereas slopes of the correlations served as indicators of differences in assay responsiveness.

The correlation coefficient ($r^2$) for the biosensor analysis of diluted raw sheep urine (measurement of parent ractopamine + intact conjugates) and the HPLC analysis of hydrolyzed sheep urine samples (parent ractopamine + hydrolyzed ractopamine conjugates) was 0.95, indicating excellent agreement between the two methods even though the biosensor measured both parent ractopamine and ractopamine conjugates, whereas the HPLC method measured free ractopamine posthydrolysis (**Figure 3**). The slope of the correlation was 1.18, indicating that the quantitative results of the biosensor were slightly greater than quantitative results obtained after HPLC analysis. **Figure 4** shows the comparison of results obtained by biosensor and HPLC analyses of enzyme-hydrolyzed sheep urine (measurement of parent ractopamine and hydrolyzed ractopamine conjugates by both assays). A correlation coefficient of 0.94 resulted, with a slope of 0.80, indicating that the biosensor yielded slightly lower quantitative results than results obtained by HPLC (concentration range = 90−9325 ng/mL, 60 samples, based on biosensor results). Collectively, these comparisons demonstrate that the biosensor generates a response to both ractopamine metabolites and free ractopamine present in urine. The discrepancies between biosensor and HPLC are small for analysis of sheep urine. When the biosensor was used to quantify ractopamine in diluted raw urine samples (sheep) and in sheep urine samples that had been pretreated with hydrolytic enzyme, the results were highly correlated ($r^2 = 0.93$), but the slope of the correlation was only 0.38 (see **Table 4**). Again, such results demonstrate that the biosensor analysis was sensitive to ractopamine conjugates. An alternate explanation is that enzymatic hydrolysis produces a matrix effect significantly different from that of unhydrolyzed urine, and although an effect of this magnitude is unlikely, it cannot be ruled out.

Correlation of the biosensor analysis with ELISA (using unhydrolyzed sheep urine for both) gave a correlation coefficient of 0.91, but the slope of the line was 0.55 (**Table 4**), indicating

**Table 3.** Comparisons of Ractopamine Quantitation by Immunosensor, ELISA, and HPLC (Nanograms per Milliliter) in Sheep Urine Samples from a Ractopamine Feeding Study

| | sheep 354 | | | | | | average of all sheep (mean ± SD) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | raw diluted urine[a] | | hydrolyzed urine[b] | | | | raw diluted urine[a] | | hydrolyzed urine[b] | | |
| day[c] | ELISA $\times 10^2$ | sensor $\times 10^2$ | ELISA $\times 10^2$ | sensor $\times 10^2$ | HPLC $\times 10^2$ | *n* | ELISA[d] $\times 10^2$ | sensor $\times 10^2$ | ELISA[d] $\times 10^2$ | sensor $\times 10^2$ | HPLC[e] $\times 10^2$ |
| T1 | 85 | 40 | 35 | 29 | 45 | 6 | 47 ± 34 | 21 ± 17 | 17 ± 12 | 16 ± 11 | 22 ± 16 |
| T2 | 119 | 72 | 45 | 41 | 59 | 6 | 82 ± 58 | 41 ± 31 | 29 ± 17 | 31 ± 20 | 38 ± 24 |
| T3 | 88 | 43 | 40 | 38 | 48 | 6 | 88 ± 35 | 46 ± 21 | 37 ± 17 | 39 ± 21 | 47 ± 18 |
| T4 | 126 | 115 | 60 | 61 | 87 | 6 | 133 ± 27 | 84 ± 25 | 48 ± 17 | 50 ± 15 | 73 ± 21 |
| T5 | 138 | 79 | 53 | 54 | 55 | 6 | 148 ± 80 | 77 ± 44 | 57 ± 30 | 57 ± 31 | 43 ± 18 |
| T6 | 88 | 55 | 41 | 33 | 44 | 6 | 108 ± 32 | 73 ± 14 | 48 ± 11 | 45 ± 12 | 51 ± 11 |
| W0 | 189 | 88 | 71 | 63 | 76 | 6 | 125 ± 37 | 63 ± 16 | 46 ± 14 | 41 ± 12 | 53 ± 14 |
| W1 | 127 | 67 | 38 | 37 | 53 | 4 | 65 ± 45 | 35 ± 24 | 23 ± 13 | 21 ± 13 | 29 ± 18 |
| W2 | 15 | 9.0 | 5.6 | 5.7 | 7.3 | 4 | 11 ± 4 | 5.4 ± 1.2 | 4.3 ± 1.5 | 3.9 ± 1.5 | 5.2 ± 1.8 |
| W3 | 4.3 | 2.0 | 1.4 | 1.6 | 2.2 | 4 | 6.5 ± 4.1 | 3.4 ± 2.1 | 2.3 ± 1.4 | 2.5 ± 1.5 | 3.3 ± 2 |
| W4 | 17 | 15 | 5.2 | 5.4 | 5.8 | 2 | 15 | 12 | 4.0 | 4.1 | 5.0 |
| W5 | 8.3 | 5.1 | 2.0 | 2.3 | 2.5 | 2 | 5.4 | 3.4 | 1.4 | 1.6 | 1.9 |
| W6 | 8.5 | 5.0 | 3.6 | 3.4 | 4.4 | 2 | 4.6 | 3.9 | 2.4 | 2.8 | 3.1 |
| W7 | 4.4 | 1.3 | 1.8 | 2.0 | 2.3 | 2 | 3.3 | 3.2 | 1.4 | 1.5 | 1.8 |

[a] Raw urine samples analyzed by cELISA and biosensor after dilution with variable amounts of 100 mM phosphate buffer containing 0.05% Tween 20 and 10 mg/mL BSA (ELISA) or HBS-EP (biosensor). [b] Urine samples were treated with enzyme to hydrolyze ractopamine conjugates, and samples were subsequently purified using liquid–liquid extraction and solid phase extraction techniques. [c] T represents day of treatment, and W represents day of withdrawal period. [d] ELISA data are from Shelver and Smith (*8*). [e] HPLC data are from Smith and Shelver (*16*).

**Table 4.** Summary of Correlations Obtained after Analysis of Sheep and Cattle Urine Samples by Optical Biosensor, ELISA, and HPLC Analyses[a]

| detector type and urine treatment | cattle | sheep |
|---|---|---|
| biosensor (diluted raw urine[b]) vs HPLC (hydrolyzed ractopamine conjugates[c]) | $y = 1.69x$ | $y = 1.18x$ |
| $y$ = biosensor result for diluted raw urine, $x$ = HPLC result for hydrolyzed conjugates in urine | $r^2 = 0.72$ | $r^2 = 0.95$ |
| biosensor (diluted raw urine[b]) vs ELISA (diluted raw urine[b]) | $y = 0.45x$ | $y = 0.55x$ |
| $y$ = biosensor result, $x$ = ELISA result | $r^2 = 0.67$ | $r^2 = 0.91$ |
| biosensor (hydrolyzed ractopamine conjugates[c]) vs HPLC (hydrolyzed ractopamine conjugates[c]) | $y = 0.74x$ | $y = 0.80x$ |
| $y$ = biosensor result, $x$ = HPLC result | $r^2 = 0.86$ | $r^2 = 0.94$ |
| biosensor (hydrolyzed ractopamine conjugates[c]) vs biosensor (diluted raw urine[b]) | $y = 0.39x$ | $y = 0.38x$ |
| $y$ = biosensor result for hydrolyzed conjugates in urine, $x$ = biosensor result for unhydrolyzed conjugates in urine | $r^2 = 0.60$ | $r^2 = 0.93$ |

[a] Samples were analyzed without hydrolysis of ractopamine conjugates or after hydrolysis of ractopamine conjugates. [b] Diluted raw urine contained ractopamine and ractopamine conjugates. [c] Urine samples were pretreated with glucuronidase/sulfatase to hydrolyze ractopamine conjugates to free ractopamine.

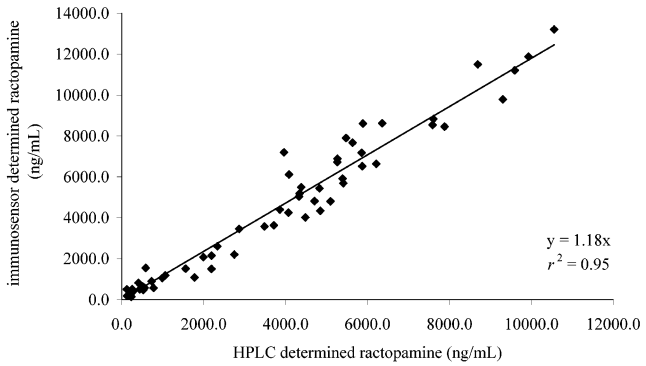


**Figure 3.** Correlation between immunosensor analyses of unhydrolyzed sheep urine samples and HPLC analysis of enzyme-hydrolyzed sheep urine samples.

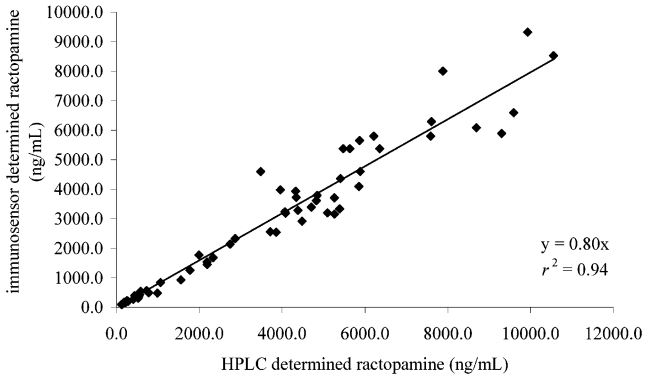


**Figure 4.** Correlation between immunosensor and HPLC measurements of enzyme-hydrolyzed sheep urine samples.

that biosensor analysis yielded smaller values than the ELISA analysis. Previously we presented evidence that quantitation of ELISA response was elevated because of significant antibody cross-reactivity with ractopamine glucuronides (*7*, *8*). Whereas the biosensor responds to metabolites, this response is less than when the same antibody is used in an ELISA format.

Regression of biosensor results for unhydrolyzed cattle urine with HPLC results yielded a correlation coefficient of 0.72 with a regression coefficient of 1.69, indicating a greater biosensor response for cattle metabolites than for sheep metabolites (**Figure 5**). Differences in the compositions of ractopamine glucuronides excreted by sheep and cattle combined with differences between the antibody selectivity for the regioconjugates of ractopamine (*7*) could explain the large difference in biosensor response in the analysis of unhydrolyzed sheep and cattle urine. Regression of results using enzyme-hydrolyzed cattle urine with both the HPLC and biosensor methods gave a correlation coefficient of 0.86 and a slope of 0.74 (range 11.8−7515 ng/mL, 63 samples). These parameters were nearly identical to the corresponding parameters calculated from the sheep urine analyses, confirming that the responses of the

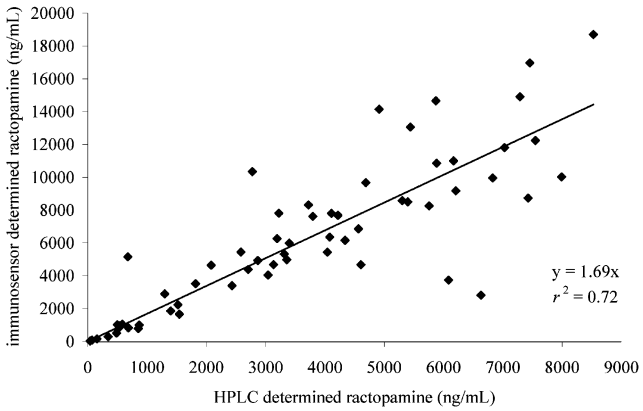


**Figure 5.** Correlation between immunosensor analysis of unhydrolyzed cattle urine samples and HPLC analysis of enzyme-hydrolyzed cattle urine samples.

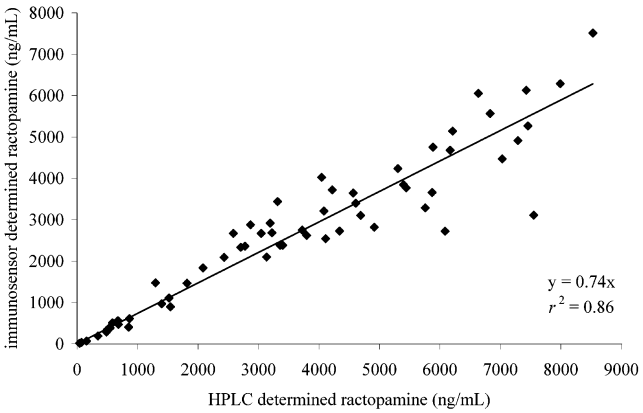


**Figure 6.** Correlation between immunosensor and HPLC measurements of enzyme-hydrolyzed cattle urine samples.

biosensor to free ractopamine were identical for both species (**Figure 6**). Regression of results obtained from biosensor analysis of unhydrolyzed (parent ractopamine + ractopamine conjugates) and enzyme-hydrolyzed cattle urine samples (unconjugated ractopamine) resulted in a correlation coefficient of 0.6 and a slope of 0.39. These results confirm the biosensor responds to ractopamine metabolites as well as free ractopamine, and the response is greater in cattle urine than in sheep urine.

When the biosensor analysis of unhydrolyzed cattle urine was compared with the ELISA analysis of unhydrolyzed cattle urine, a correlation coefficient of 0.67 and a slope of 0.45 resulted. Thus, the ELISA had a greater response to ractopamine metabolites than did the biosensor. Additionally, this response was greater for cattle urine than for sheep urine, for which a slope of 0.55 and a correlation of 0.91 were observed. Consistent with sheep urine, the biosensor method was less sensitive to metabolites than the ELISA. Even though the same antibody was used for the biosensor and the ELISA, the formats of the assays are very different. In the biosensor the interactions are basically kinetically controlled with the antibody interacting with the analyte immobilized on the chip, whereas the ELISA involves essentially thermodynamically controlled interactions between the analyte and the antibody immobilized on the well. Consequently, the processes are fundamentally quite different and would be expected to interact with analytes differently. Furthermore, the difference between cattle and sheep would be expected because previous ELISA experiments showed significant differences in the analysis of incurred urine samples from the two species. Although other explanations are possible, differences in metabolism changing the relative amounts of conjugated metabolites could produce the observed results. The antibody shows different sensitivities toward various metabolites; consequently, the response would vary if the samples had diverse conjugated metabolite compositions. Despite the significant differences in the results, the differences are relatively small, indicating similarities between the two assays despite the variation in format.

Results obtained from the biosensor were comparative to ELISA and HPLC methods when ractopamine conjugates present in urine were hydrolyzed prior to analysis. For HPLC analysis of ractopamine, urine samples require additional liquid−liquid and solid phase extraction prior to analysis, making the analysis complex. The biosensor and ELISA methods offer advantages of ease of sample preparation and high throughput. The biosensor method appears to be less subject to matrix effects, or at least these effects are smaller than we have observed in ELISA methods. The biosensor analysis, similar to ELISA, increased capacity for detection for urinary ractopamine by two withdrawal days past which the HPLC method limit of quantitation (LOQ) had been reached. The increased sensitivity of the biosensor analysis was probably due to the detection of metabolites, making the method an excellent choice for detecting illegal use of ractopamine.

In conclusion, a stable, reproducible, and sensitive method of analyzing ractopamine in urine samples using biosensor technology has been developed. This technology allows the automated and rapid analysis of samples for ractopamine content. Although the same antibody was used for both the ELISA and the biosensor, the responses to samples were significantly different, with the ELISA being more sensitive to metabolites, probably due to differences in how the signal is generated in these two techniques. Although the ELISA was slightly more sensitive depending on the extent of metabolism, the biosensor analysis is more convenient, readily automated, and an excellent alternate method of analysis. Both ELISA and the biosensor analysis require an enzyme deconjugation step to determine total ractopamine.

## ABBREVIATIONS USED

BSA, bovine serum albumin; EDC, *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); HPLC, high-performance liquid chromatography; LOQ, limit of quantitation; NHS, *N*-hydroxysuccinimide; SPE, solid phase extraction; SPR, surface plasmon resonance.

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